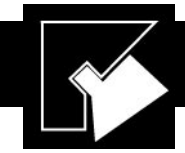
AFFINITY SEPARATION



Affinity Membranes

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The rapid development in biotechnology and the large potential of biomolecules for applications in medicine, food industry and other areas, result in an increasing demand for efficient and reliable tools for the purification of proteins, peptides, nucleic acids and other biological substances. This situation is being additionally enforced by the increasing number of recombinant gene products that have arrived on the market or that are currently being investigated, such as insulin, erythropoietin and interferons. The recovery of fragile biomolecules from their host environments requires their particular characteristics to be taken into account for the development of any extraction or separation process. On the other hand, there is a demand for techniques that can easily be scaled up from laboratory to industrial production level.

In this context, the use of affinity methods has the advantage that coarse and fine purification steps are united through the introduction of a specific recognition phenomenon into the separation process. The most widely used method for preparative affinity separation of biomolecules is liquid chromatography on beaded resins (soft gels). Despite the commercial availability of many affinity ligands immobilized on to gel beads for use in column chromatography, there are some drawbacks in a large scale application of these supports. Flow rates and thus performance are limited by the compressibility of the resins and pore diffusion. Because of these intrinsic limitations, other chromatographic techniques, such as perfusion chromatography, or different separation techniques, such as affinity precipitation and affinity phase partitioning, have been suggested as possible alternatives. Another technique that is gaining increasing importance is membrane-based separation. Adsorptive membrane chromatography was introduced as a purification method in the mid 1980s. Microporous membranes have been successfully coupled with biological or biomimetic ligands, yielding affinity membrane chromatography supports. Several of them, with for example protein A and G, dye or metal chelate ligands, are commercially available. Affinity membrane chromatography is in fact a hybrid technique combining affinity gel chromatography and membrane filtration, with the advantages of the two technologies.

The purpose of the present review is to discuss relevant aspects and developments that are important for the design of an affinity membrane chromatography process, including the choice of the membrane material, coupling chemistry, affinity ligands, membrane configurations, operation modes and scale-up. In a wider sense, membrane-based affinity fractionation also comprises affinity filtration methods where the target molecule binds to an affinity ligand coupled to nanoparticles, which can then be separated by filtration through a membrane. However, this application will not be discussed here in detail.

General Characteristics of Membrane Chromatography

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In contrast to chromatographic supports based on beaded resins with dead end pores, membrane chromatographic supports have through-pores and lack interstitial space. Mass transfer is mainly governed by forced convection and pore diffusion is negligible. The observed back-pressures are normally quite low, and high flow rates and thus high throughputs and fast separations become possible without the need for high pressure pumps or equipment. As the association time for an antibody–antigen complex is typically about 1 s or less, but the diffusion of a protein molecule to the centre of a 50 μm porous bead takes tens of seconds, in a membrane support, the low diffusional limitation leads to faster adsorption kinetics and higher throughput efficiency. Little deterioration of the separation efficiency occurs even at elevated flow rates. On the other hand, with affinity membranes the formation of the affinity complex can become the rate-limiting process at high flow rates.

A problem often encountered in membrane chromatography is extra-cartridge back-mixing, which can severely degrade membrane performance. This phenomenon is due to dead volumes outside the membrane, in tubing, fittings and valves, and leads to peak broadening and dilution. It is more pronounced

in membrane chromatography systems compared to conventional columns packed with beaded supports, owing to the larger throughput/bed volume ratio.

Although the specific surface area of membranes is typically only 1% of that of conventional chromatographic resins, microporous membrane systems have high internal surface areas and reasonably high capacities. The open-pore structure of membranes increases the accessibility of affinity ligands and reduces steric hindrance compared to small-pore adsorbents.

Membrane Geometry

Just like filtration membranes in general, affinity membranes can be produced in different configurations, and membrane modules of various geometries are commercially available or have been manufactured in research laboratories (**Figure 1**).

Flat sheet or disc membranes can be mounted as individual membranes in specially designed cartridges or in commercial ultrafiltration units for use in dead-end filtration mode. This allows for the production of inexpensive single- or multiple-use devices for the rapid adsorption of a target molecule from dilute samples in batch or continuous recycling mode. Cartridges are also available that allow for operation in cross-flow filtration mode.

Stacks of flat membrane discs have been employed for affinity membrane chromatography in column-like devices, the main purpose being to increase the adsorption capacity. Another configuration is continuous rod-type membranes which can be directly cast in a chromatographic column. Both types of membrane columns are compatible with conventional high performance liquid chromatography or fast protein liquid chromatography systems and have advantages over columns packed with beaded resins, as described above. Being highly porous with a mean pore diameter of 0.1–10 μm, they allow for efficient separations even at high flow rates.

If the target molecule is to be recovered from complex feed solutions such as cell homogenates or blood plasma, or from solutions containing high molecular mass additives such as antifoam agents or even particulate material, the use of membranes in dead-end filtration mode is often impossible due to membrane fouling. A remedy to this problem is the operation in cross-flow filtration mode where the build-up of a polarization layer at the membrane surface is avoided or diminished. Hollow-fibre membranes are well adapted for such applications. They are usually

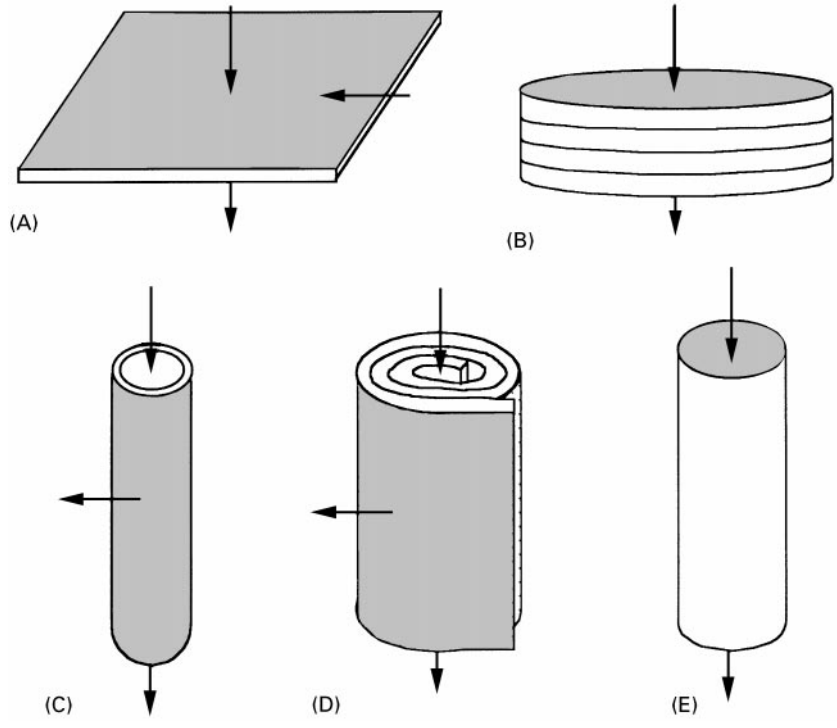

Figure 1 Different geometries of affinity membranes. (A) Flat sheet; (B) stack of flat discs; (C) hollow fibre; (D) spiral-wound flat sheet; (E) continuous rod. The arrows indicate flow directions. (Adapted from *Journal of Chromatography* 702, Roper DK and Lightfoot EN, Separation of biomolecules using adsorptive membranes, pp. 3–26, Copyright 1995, with permission from Elsevier Science.)

mounted as bundles in tubular cartridges. Another configuration are flat-sheet membranes that are spiral-wound around a cylindrical core. Both systems have the advantage of high surface area/cartridge volume ratios and high operational capacities.

Membrane Material, Activation and Ligand Coupling

Membrane Material

Due to the specific properties of biomolecules, the membrane materials to be used for their separation should ideally possess the following characteristics:

- Macroporosity: This will allow biomolecules to cross the membrane and to access the affinity sites.
- Hydrophilicity: Using hydrophilic supports, nonspecific adsorption by hydrophobic interactions and denaturation of biomolecules can be avoided.
- Presence of functional groups: These are required for the coupling of an affinity ligand.
- Chemical and physical stability: The material has to withstand the sometimes harsh conditions during derivatization, operation and regeneration.
- Biocompatibility: This is particularly important if the membranes are used in extracorporeal devices, for example for blood treatment.
- Large surface area relative to membrane volume: This will allow for the construction of small, integrated devices with high operational capacities.

Cellulose and cellulose acetate were among the first materials that have been used for affinity membrane preparation. They are hydrophilic and biocompatible, and due to the presence of hydroxyl groups, ligand coupling can be easily achieved using for example CNBr or carbonyldiimidazole activation. In order to improve the mechanical and chemical stability of cellulose membranes, chemical cross-linking with epichlorohydrin is sometimes carried out. Cellulose membranes normally have a rather small pore size, resulting in a high pressure drop. Attempts to produce membranes with larger pores using coarse cellulose fibres have resulted in a less uniform membrane structure.

Polysulfone is another suitable membrane material which has good film-forming properties. It is of sufficient physical, chemical and biological stability, and ligands can be coupled after chloromethylation-amination or acrylation-amination.

Microporous polyamide (nylon) membranes have also been used for the preparation of affinity membranes. This material is mechanically stable and has a rather narrow pore size distribution. It contains only a small number of terminal amino groups for ligand coupling, which can, however, be increased by partial hydrolysis of the amide functions.

A suitable membrane material is polyvinyl alcohol, in particular because of its hydrophilicity and biocompatibility. Poly(ethylene-co-vinyl alcohol), which has a somewhat higher chemical stability, has also been used. Both materials contain hydroxyl groups and can be activated by the CNBr method, allowing immobilization of affinity ligands having an amino function. Ligands can also be coupled using epichlorohydrine or butanediol diglycidyl ether-activation.

Other materials that have been used for affinity membranes are poly(methyl methacrylate), poly(hydroxyethyl dimethacrylate), polycaprolactam, poly (vinylidene difluoride), poly(ether-urethane-urea) and silica glass. **Table 1** shows a list of membrane materials and the appropriate ligand-coupling chemistries.

Composite Membranes

The main difficulty when choosing a membrane for affinity separation of biomolecules is sometimes to find a material that fulfils several or all of the above-mentioned requirements. For example, a chemically stable material might be too hydrophobic and lead to nonspecific and irreversible adsorption of the protein to be separated, whereas a hydrophilic material that is compatible with the fragile protein molecules might not withstand the conditions required for ligand coupling and for regeneration and sterilization of the membrane. Therefore, the choice of a membrane material will sometimes be a compromise. The use of a composite membrane consisting of two or more different materials may often be the only solution to a particular separation problem. This approach consists of the grafting of hydrophilic polymers on to a chemically and mechanically stable microporous membrane. The result is an increased biocompatibility as well as the introduction of suitable functional groups for ligand coupling. One example is the radiation-induced graft polymerization of 2-hydroxyethyl methacrylate or glycidyl methacrylate on to a polyethylene hollow fibre membrane. This increases the hydrophilicity of the material and introduces active hydroxyl groups or reactive epoxy groups.

Activation and Ligand Coupling

From a practical point of view, apart from the chemical compatibility of the membrane material with the activation and coupling solutions, an important

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Table 1 Membrane materials and possible chemistries for ligand coupling

Membrane material	*Coupling chemistries*	*Ligand functional group*
Cellulose, cellulose diacetate	Epichlorohydrin, butanediol diglycidyl ether	Amino, (hydroxyl)
	Carbonyldiimidazole	Amino, hydroxyl
	CNBr	Amino
	Anhydride	Amino
	Hydrazide	Amino
Polysulfone	Acrylation-amination, chloromethylation-amination	Cl^- (aromatic)
	Ethylene glycol diglycidyl ether	Amino, (hydroxyl)
Polyamide	Glutaraldehyde (Schiff base)	Amino, amido
	Divinyl sulfone	Hydroxyl
	2-Fluoro-1-methylpyridinium toluene-4-sulfonate	Primary amino
	Butanediol diglycidyl ether	Amino, (hydroxyl)
	Formaldehyde	Hydroxyl
Poly(vinyl alcohol), poly(ethylene vinyl alcohol)	Epichlorohydrin, butanediol diglycidyl ether	Amino, (hydroxyl)
Electrostatically spun poly(ether-urethane-urea)	Carbonyldiimidazole	Amino, hydroxyl
	CNBr	Amino
	Carbonyldiimidazole	Amino, hydroxyl
Glass	Glycidoxypropyl trimethoxysilane	Amino, (hydroxyl)
	Aminopropyltrimethoxysilane	Carboxyl

aspect is that these solutions need to access the pores of the membrane. In many cases it will therefore be necessary to do the activation in dynamic mode, that is, by forced convection. This is especially important if the membrane material is hydrophilic and the activation and coupling solutions are based on nonpolar solvents, since in that case the wettability of the membrane by the solutions will be low.

Spacer Arms

Occasionally, affinity membranes may show poor performance if the ligand, and in particular a small ligand, is coupled directly to the membrane. This is often due to a low steric availability of the ligand, a problem that can be overcome by the use of a suitable spacer arm. In that way, the ligand accessibility for the molecule to be separated is improved, resulting in an increase in membrane-binding capacity. For example, 1,6-diminohexane or 6-aminohexanoic acid are often used as spacers. In other cases, the coupling method itself provides a spacer, as is the case with butanediol diglycidyl ether. If composite membranes with crafted flexible copolymer chains are used, spacer arms are not normally required.

Affinity Ligands

Biologicial Ligands

Just like other affinity separation techniques, affinity membrane technology uses biomolecules as the affinity ligands, thus taking advantage of the specificity of biological recognition. One of the most common applications is the use of immobilized monoclonal antibodies against natural or recombinant proteins as the ligand for immunoaffinity separation. Another important example are membranes with covalently coupled protein A or protein G for immunoglobulin purification from plasma, serum or cell culture supernatants. Immobilized lectines have been used for the purification of glycoproteins. The use of inhibitors or coenzymes for the purification of enzymes is also possible. Although biomolecules are widely used as ligands for their selectivity, they do have drawbacks. Their poor stability and sometimes high price can make them problematic for use in large scale affinity separation. Drastic conditions are often necessary for elution of the ligate, for example with high affinity antibody–antigen interactions. This can lead to partial inactivation of the molecule to be purified. Ligand denaturation and inactivation, in particular with protein ligands, can occur during regeneration and sterilization of the membrane. Another important issue is the possible leaching of the affinity ligand, leading to a contamination of the final product, which is particularly problematic if the product is to be used in medical applications.

Pseudobiospecific Ligands

An alternative approach involves the use of biomimetic or pseudobiospecific affinity ligands. These are usually smaller and simpler molecules with higher chemical and physical stability than biomolecules. The working principle of pseudobiospecific ligands relies on the complementarity of

structural features of ligand and ligate rather than on a biological function, whereas biomimetic ligands have a certain structural resemblance with a biological ligand. For example, textile dyes can be used for the separation of proteins, and in particular Cibacron Blue F3GA has been employed as ligand in affinity membranes for the purification of dehydrogenases, since it often binds specifically to the nucleotide-binding site. Other dyes may adsorb proteins less specifically, but by selection of the right dye (a large number of different dyes is currently available) and the appropriate adsorption and elution conditions, highly efficient separations can be obtained.

Proteins carrying accessible histidine residues on their surface have been shown to have affinity for transition metal–chelate ligands. Typical examples are the iminodiacetate–copper(II) complex (IDA-Cu(II)) and the nitrilotriacetate–nickel (NTA-Ni(II)) ligand widely used for purification of recombinant proteins with genetically attached poly-His tails.

A third group are amino acids such as phenylalanine, tryptophane and histidine. Being the least selective, they have nevertheless been successfully employed for protein purification. However, fine-tuned adsorption and elution conditions are necessary to achieve efficient separation. Mention should also be made of the thiophilic affinity system that has been used with affinity membranes. It is based on the salt-promoted adsorption of proteins via thiophilic regions (containing aromatic amino acids) on to sulfone or thioether-containing heteroaliphatic or aromatic ligands.

Molecularly Imprinted Membranes

A completely different approach for the preparation of affinity membranes is the use of molecularly imprinted polymeric materials. These are produced by polymerization of functional and cross-linking monomers in the presence of the target molecule (the molecule to be separated later), which acts as a molecular template. In this way, binding sites are introduced in the polymer that are complementary in shape and functionality to the target molecule, and that often have specificities comparable to those of antibodies. At the same time, the cross-linked polymeric material provides a porous, chemically and physically very stable support. Even though the technology is in principle applicable to larger biomolecules such as proteins, it has mainly been used for the separation of small molecules like amino acids and peptides. The molecular imprinting technique is reviewed in more detail elsewhere.

Scale-up

Process scale-up tends to be rather easy in adsorptive membrane chromatography, at least compared to the use of conventional beaded resins as the chromatographic support. It has been demonstrated that the diameter of a stack of disc membranes can be increased by up to one order of magnitude and more, with the dynamic capacity remaining constant. This allows for the processing of considerably larger sample volumes at higher flow rates. With radial flow membranes, when both the height and diameter of the cartridge were increased and the flow rate adjusted proportionally to the increased cartridige volume, the apparent specific capacity decreased only slightly.

Applications

Several different applications of affinity membranes have been described. Typical examples of their use for the separation and purification of biomolecules are shown in **Table 2**.

The most common application is the separation and purification of biomolecules and especially proteins for large scale production. A common example is the separation of immunoglobulins from blood-serum or plasma or from cell culture supernatants. Hollow-fibre cartridges with immobilized protein A or pseudobiospecific ligands have been used for this purpose. **Figure 2** shows a chromatogram from a case study of immunoglobulin G separation from human plasma using a small, developmental-scale (28 cm^2 surface area) poly(ethylene-co-vinyl alcohol) hollow-fibre membrane cartridge. The pseudobiospecific affinity ligand histidine was immobilized on to the membrane after activation with butanediol diglycidyl ether, thus introducing a spacer arm. Serum was injected 10-fold diluted in cross-flow filtration mode. Weakly retained and entrapped proteins were then removed by washing the lumen and the outer shell of the fibres, as well as the pores in back-flushing mode. Adsorbed immunoglobulins were subsequently eluted with a buffered solution of 0.4 mol L^{-1} NaCl in back-flushing mode. The eluted fraction contained 93% immunoglobulins (82% IgG, 10.8% IgM). The dynamic binding capacity of the membrane for immunoglobulin G was determined to be 1.9 g m^{-2}. The process could then be scaled up by using a cartridge with 1 m^2 membrane surface area.

A related application is the final polishing of an already pure product. For example, the removal of bacterial endotoxins from contaminated solutions of monoclonal antibodies has been demonstrated using membrane-bound pseudobiospecific ligands.

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Table 2 Examples for the use of affinity membranes for isolation and purification of biomolecules

Isolated substance	*Affinity ligand*	*Membrane material*	*Configuration*	*Application*
Human serum amyloid protein	Anti-hSAP Ab (polyclonal)	Cellulose	Flat sheets	Extracorporeal circuit, removal of amyloid from blood
Heparin	Poly-L-lysine	Cellulose diacetate poly(ethylene-co-vinyl alcohol), coated polyethylene	Hollow fibres	Extracorporeal circuit, removal of heparin from blood
Human IgG	Recombinant protein A	Poly(caprolactam) Modified poly(caprolactam) Polysulfone-coated hydroxyethyl cellulose	Hollow fibres Hollow fibres, flat sheet Hollow fibres	Purification
Recombinant protein G	Human IgG	Glycidyl methacrylate-co-ethylene dimethacrylate	Discs	Purification
Trypsin (porcine)	Soybean trypsin inhibitor	Modified cellulose	Spiral wound sheet (radial flow)	Purification
Glucose-6-phosphate dehydrogenase	Cibacron blue	Nylon	Stack of flat sheets	Purification from clarified yeast homogenate
Human IgG Autoantibodies	Histidine	Poly(ethylene-co-vinyl alcohol)	Hollow fibres	Purification from blood plasma and serum Removal from blood plasma in extracorporeal circuit
Lysozyme, cytochrome *c*, ribonuclease A	IDA-Cu^{2+}	Glass	Hollow fibres	Purification

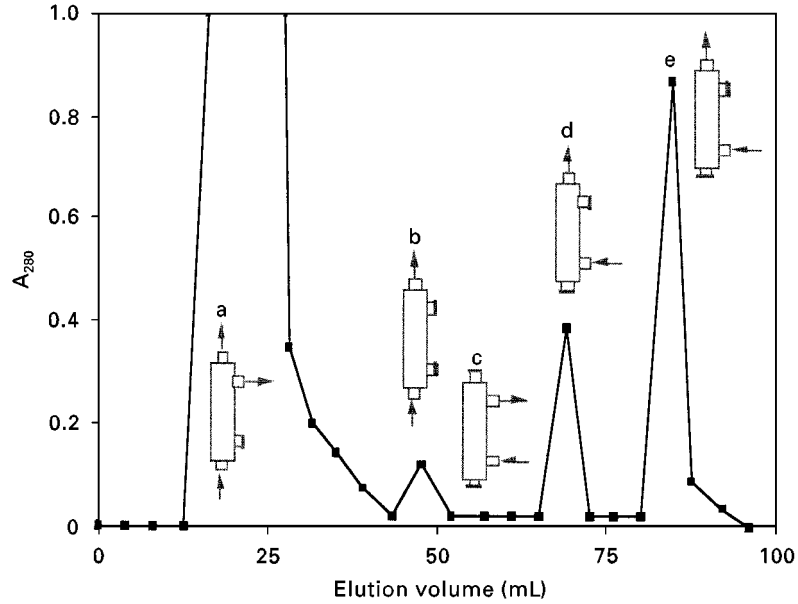

Figure 2 Separation of immunoglobins from human serum using a poly(ethylene-co-vinyl alcohol) hollow-fibre cartridge with immobilized L-histidine. (a) Immunoglobulin adsorption in cross-flow filtration mode; (b) lumen wash; (c) shell wash; (d) back-flush wash; (e) back-flush elution. (Adapted from *Journal of Membrane Science* 117, Bueno SMA, Legallais C, Haupt K and Vijayalakshmi MA, Experimental kinetic aspects of hollow-fiber membrane-based pseudobioaffinity filtration: Process for IgG separation from human plasma, pp. 45–56, Copyright 1996, with permission from Elsevier Science.)

Affinity membranes have also been suggested for use in extracorporeal circuits, for the removal of toxic substances such as certain metabolites or antibodies from blood. For example, exogenous human serum amyloid P component, a substance associated with Alzheimer's disease, has been removed from whole rat blood in an extracorporeal circulation system. This model system used a polyclonal antibody coupled to cellulose flat-sheet membranes. The biocompatibility of the membrane was also demonstrated. A similar application is the removal of autoantibodies from human plasma, using membrane-bound affinity ligands in extracorporeal circuits.

Apart from preparative applications, small cartridges with membrane discs or continuous membrane rods should be useful for analytical-scale separations and affinity solid-phase extraction, for example for immunoextraction.

Conclusions

Affinity membrane separation techniques combine the specificity of affinity adsorption with the unique hydrodynamic characteristics of porous membranes. They provide low pressure separation systems which are easy to scale up and ideal for the processing of large volumes of potentially viscous feed solutions (e.g. microbial broth, bacterial cell extract, conditioned media) often involved in the production of recombinant proteins. The additional microfiltration effect of membranes allows for the processing even of unclarified, particle-containing feed solutions. The high performance of this separation technique is due to the presence of through-pores and the absence of diffusional limitations; mass transfer is mainly governed by forced convection. Affinity membranes are used in applications such as purification of biomolecules, final product polishing, removal of unwanted substances from patients' blood in extracorporeal circuits, but also for smaller scale analytical separations. Biological affinity ligands and biomimetic or pseudobiospecific ligands are currently employed, as well as different membrane configurations such as flat sheets, hollow fibres or continuous rods. The technology is now in the process of being adapted more and more for large scale industrial separation and purification.

See also: **I/Affinity Separation. Membrane Separations. II/Affinity Separation:** Dye Ligands; Immunoaffinity Chromatography; Imprint Polymers; Rational Design, Synthesis and Evaluation: Affinity Ligands; **Chromatography: Liquid:** Large-Scale Liquid Chromatography. **Membrane Separations:** Filtration. **III/Immunoaffinity Extraction. Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Properties. Essential Guides for Isolation/Purification of Immunoglobulins. Appendix 2/Essential Guides to Method Development in Affinity Chromatography.**

Further Reading

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Affinity Partitioning in Aqueous Two-Phase Systems

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Aqueous Two-phase Systems in General

The division of water into non-miscible liquid layers (phases) by addition of two polymers has led to the remarkable possibility of being able to partition proteins and other cell components between phases of nearly the same hydrophilicity. Proteins can be separated by partitioning if they have unequal distribution between the phases, i.e. when their partition coefficients, K (the concentration in top phase divided by the concentration in bottom phase), differ. Usually the difference in the K value of many proteins is not very large and then repeated extractions have to be carried out to get a reasonable